52 Protozoa

J. D. STOUT

Soil Bureau, Department of Scientific and Industrial Research
Lower Hutt, New Zealand (deceased 3 March 1982)

S. S. BAMFORTH

Newcomb College, Tulane University
New Orleans, Louisiana

J. D. LOUSIER

Research Branch, Ministry of Forests
Victoria, British Columbia, Canada

52–1 INTRODUCTION

Four main taxonomic groups of Protozoa are recognized: the amoebae (naked and testate), the flagellates, the ciliates, and the wholly parasitic sporozoa (Grell, 1973; MacKinnon & Hawes, 1961). Since symbiotic or parasitic protozoa are normally associated with many soil animals, the infective stages must pass through the soil. These include the multiflagellate Hypermastigida found in the gut of termites, some of which can digest cellulose, the sporozoan Gregarinida, infecting the seminal vesicles of earthworms, and symbiotic ciliates found in the gut of earthworms. Most arthropods may carry protozoa externally, or as internal parasites, and some protozoa infect plants. Thus, protozoa occurring in the soil or associated with other soil organisms include a very wide range of types, and no one technique will reveal them all (Table 52–1).

In this chapter, techniques adapted to study free-living populations are evaluated. However, traditional approaches have generally proved inadequate, and improvement and further testing are needed. Methods must be experimented with and adapted to the particular soil and protozoan group being studied.

The free-living populations are not evenly distributed throughout the soil. They normally tend to be associated with decaying plant debris near the soil surface and are more common near the rhizosphere than away from it (Bamforth, 1976), but there are a large number of free-living species, and

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Table 52–1. Principal free-living soil protozoan groups.

A. Small flagellates
   Ubiquitous: e.g., *Oikomonas* (one flagellum), *Bodo* (two flagella, one trailing)

B. Naked amoebae
   Ubiquitous
   1. Small monopodal: *Vahlkampfia, Naegleria, Hartmannella* (including parasitic species)
   2. Multipodal: *Nuclearia*
   3. Pellicle layer: *Thecamoeba*
   4. Slime molds: *Dictyostelium*

C. Ciliates
   Reflect soil structure, moisture, and aeration
   1. Trichostomes: Almost ubiquitous; e.g., *Colpoda,* oval flat body with indented cytostome; *Leptopharynx,* small flat with coarse cilia in furrows
   2. Small edaphic ciliates
      Chilodonella, humped, with cilia restricted to ventral surface
      Cyrtolophosis, ovoid, with cytostome in depression in anterior end
   3. Larger and more complex hypotrichs; *Uroleptus, Keronopsis, Gonostomum*
   4. Sessile peritrichs, e.g., *Vorticella striata*
   5. Predatory species, *Spathidium, Bresslaua*
   6. Histophage, *Tetrahymena rostrata* (facultative parasite)
   7. Suctoria, sessile adult with ciliated larva, e.g., *Podophrya*

D. Testacea
   The shape of the test commonly reflects the soil moisture regime
   1. Globose or hemispherical tests, e.g., *Cyclopyxis, Phryganella*
   2. Oval, with subterminal aperture, e.g., *Trinema, Corythion, Centropyxis*
   3. High-vaulted tests, normally associated with forest litters and mosses, e.g., *Nebela, Difflugia*
   4. Flattened, plano-convex in profile tests, normally in open structures, e.g., litter (*Arcella, Microchlamys*)

they differ widely in their habitat preference. This preference is related to size or shape of the species; ability to encyst or to move through the soil; nutrition; or tolerance of adverse environmental factors, e.g., high salinity, acid pH, low O_2 or high CO_2 tensions. The most ubiquitous species are small (<10 μm in diam), feed on bacteria or dissolved organic nutrients, encyst and excyst readily, and are able to move freely through the soil. They include the small saprozoic flagellates and the naked amoebae. As a morphological adaptation to soil conditions, the more common Testacea tend to be small (<45 μm), with small or partly concealed mouth openings. Larger and more elaborate species are more often associated with the humid conditions of the forest floor.

Food supply and diversity as well as moisture limit population size, distribution, and species composition, and not all populations peak at the same time of the year or in the same soil horizon. Estimates of populations of flagellates, amoebae, and cilites are summarized by Heal (1971) and in Table 52–2, and estimates of Testacea are given in Table 52–3.

In this chapter, we discuss (i) culture methods for free-living protozoa, (ii) counting methods, and (iii) examination methods. It is important to have some familiarity with the soil being studied and its fauna before techniques are selected for estimating populations, and these should be adapted for the purposes of the study.

52-2 CULTURE METHODS

52-2.1 Principles

Different methods are required for different soils and for different protozoan groups. Cultures are selective and may be chosen to reveal either a broad spectrum of species or a specialized group. Cultures enriched with nutrients will be more selective than those dependent solely on the nutrients contained in the soil, and fewer species will grow on an agar surface than in a liquid medium. Liquid over an agar surface normally provides the most suitable medium. One convenient technique to encourage the growth of bacteria-feeding protozoa is to add an inoculum of an edible bacterium. However, although this will promote the growth of predacious protozoan species, it will also stimulate the growth of other, and perhaps inedible, bacteria.

For routine examination of a normal soil, cultures should be set up to grow a broad spectrum of species. Subsequently, subculturing can establish a pure culture of a single protozoan species. Techniques are described by Heal (1970) and Galtsoff et al. (1959). The most numerous species may also be isolated into pure culture by serial soil dilution. Finally, a pure culture may be made axenic, i.e., free of other organisms such as bacteria, by the use of antibiotics and provision of a suitable nutrient medium (MacKinnon & Hawes, 1961). However, axenic cultures of some protozoa, notably the Testacea, are very difficult to establish. Pure culture is sometimes necessary to establish the identity of the species and axenic culture is necessary to determine its physiology.

52-2.2 Aerobic Protozoa

The most convenient medium is a firm agar (2% of a good commercial agar) in a 10-cm petri dish. Add 10 g (wet wt) of soil to the surface of the agar on one side of the dish. Add 20 ml of sterile distilled water carefully to the other side. By keeping the soil to one side, the protozoa are able to migrate to the clear translucent part of the dish and can be observed directly under the microscope with a dry or water immersion lens. Distilled water enhances excystment, and the soil supplies the nutrients necessary for the bacteria, which grow as a film over the agar surface. Incubation temperature can be varied within limits. Growth is most rapid at higher temperatures ($>20°C$) but may be inhibited above 25°C. Below 10°C some protozoa may not grow or excyst. The most suitable temperature, therefore, is from 15 to 20°C.

Normally, small flagellates appear first, followed in a few days by small amoebae. Ciliates and Testacea occur later. Nematodes, rotifers, tardigrades, or microdrili can occur in these cultures.

Cultures may be enriched with soil extract or nutrient agar (Heal, 1964, 1971). This will increase the populations of some species and prevent the

growth of others. Alternatively, a suspension of edible bacteria (e.g., *Enterobacter cloacae*) may be added or streaked across the agar plate before soil inoculation. This will select fast-growing bacteria-feeding species, such as the small amoebae or the ciliate *Colpoda.* Additional species of amoebae will often appear if a weak saline solution and grain infusion or soil extract agar are used (Page, 1976). Reducing or deleting the water overlying the agar inhibits competitive flagellates and ciliates.

52–2.3 Anaerobic Culture

Strictly anaerobic species are rare outside the animal gut, but there is a small group of ciliates, known as the *sapropelic fauna*, found in sewage or wet, organically rich sites. More common are ciliates, such as *Metopus,* which tolerate traces of O_2. The simplest technique for culturing soils in which they may occur is to use a liquid medium, such as soil extract, in a screw-capped container (20 ml). Because they are normally organically rich, it is not necessary to enrich the medium to ensure anaerobic conditions. Alternatively, the normal agar plate culture may be incubated in McIntosh and Fildes jars or another type of anaerobic incubation.

52–2.4 Culture by Serial Dilution

A modification of the simple petri dish technique has been developed for dilution culture. A number of cultures are set up on the same dish. Two modifications are used; either a ring of bacterial inoculum is prepared within which the protozoan culture is placed, or a glass or plastic ring is sunk into the agar and the protozoan culture set up within it. The former modification is useful only for bacteria-feeding protozoa. The latter is less liable to dry out and permits a wider range of protozoa to be cultured. Isolation by the dilution technique permits slow-growing species to be established in pure culture and is particularly valuable for Testacea.

52–2.5 Overlay Technique

A convenient culture technique for evaluating populations of small amoebae has been developed by Menapace et al. (1975). A nonnutrient agar is poured into a 20 by 100 mm petri dish and allowed to solidify. It is inoculated from soil dilutions that are spread with a glass rod. A mineral salt agar with phosphate buffer and the addition of a suitable edible bacterium is used as an overlay. The salts used are 4.0 m*M* of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 50.0 μM of ferrous sulfate ($FeSO_4$), 5.0 m*M* of potassium monohydrogen phosphate (K_2HPO_4), 5 m*M* of potassium dihydrogen phosphate (KH_2PO_4), and 2.5 m*M* of ammonium sulfate [$(NH_4)_2SO_4$], with 0.8% (wt/vol) agar, and *Enterobacter aerogenes* is the bacterial inoculum. The plates are incubated for 7 to 9 days at 30°C, and the clear zones indicate the growth of the amoebae. This technique, however, probably underestimates amoeba numbers and may prove better without the overlay (O'Dell, 1979).

52–2.6 Methods for Slime Molds (Acrasieae)

The cellular slime molds differ from most free-living protozoa in having an elaborate life history with active trophic amoebae, plasmodia, fruiting bodies, spores, and microcysts (Grell, 1973). Singh (1947) used his bacterial ring technique for isolating them from soil. Cavander and Roper (1965) developed a technique involving preliminary rotary shaking of the soil sample to separate the slime molds from the soil. This technique was biased in favor of spores and microcysts, since amoebae were killed by the rotary shaking. Kuserk et al. (1977) have modified the technique by moistening the soil sample with Bonner's salt solution and gently agitating the suspension. One portion is plated on cerophyll agar, with a suspension of *Escherichia coli* added to serve as food. The culture is incubated at 23°C on a 12:12 light/dark cycle and clones counted as they appear. A second portion of the suspension is frozen for 24 hours, thawed, and plated. The difference between the two platings provides an estimate of the active amoebae.

52–3 COUNTING METHODS

52–3.1 Introduction

Populations are estimated by three means: directly observing the protozoa in the sample, mounting them on a slide (Jones-Mollison technique) or suspending them on a Millipore filter (Coûteaux technique), or by serially diluting and subsequently growing them in culture (most probable number [MPN] technique). All are subject to error, and their suitability must be assessed in relation to the particular soil being sampled, the populations being studied, and the purpose of the investigation (Meisterfeld, 1979). It is important to distinguish the biological significance of the data from their statistical significance. Generally, direct observation is used for Testacea, and MPN technique is used for flagellates, amoebae, and ciliates.

Attempts to extract protozoa from the soil have been made with various techniques but with only limited success. A number of techniques involving bubbling of gas through a soil suspension so as to float testacean shells to the surface have been developed (Bonnet, 1964; Bonnet & Thomas, 1958; Chardez, 1959, 1964; DeCloitre, 1960, 1966; Laminger, 1972). However, these techniques are inadequate for quantitative work (Korganova & Geltser, 1977; Lousier, 1979[1]), have been shown to be inefficient (Coûteaux, 1967), and can render tests unidentifiable because of microscopic distortion (Lousier, 1979[1]). Other laboratory separation techniques are not generally applicable to soil samples (Claff, 1940). Extraction of active ciliates by electromigration (Hairston, 1964; Borkott, 1975), temperature (Uhlig, 1964). or centrifugation (Berk et al., 1976) shows con-

[1] J. D. Lousier. 1979. Population ecology of Testacea (Protozoa, Rhizopoda) in an aspen woodland soil. Ph.D. Thesis. Univ. of Calgary, Calgary, Alberta, Canada.

siderable potential, but these procedures have not been adequately developed. Similarly, because of the difficulties of separating the protozoa from the soil, little use has been made of particle counters (Borkott, 1975). Direct enumeration of ciliate protozoa has been used for benthic samples, however, and should be used for suitable soils (Finlay et al., 1979).

52–3.2 Direct Observation

The most widely used direct observation technique has been the agar film technique described by Jones and Mollison (1948). In this method (c.f. section 44–3.1.1), a soil suspension amended with agar is molded into films of constant and known thickness and mounted on a microscope slide. One gram of material is ground in a small beaker with 5-ml aliquots of distilled water and added to 25 ml of 1.5% liquid agar. Samples are pipetted onto a hemacytometer and the slide immersed in ice water. The solidified film is floated off onto a glass slide, air-dried, and stained for 1 hour in a solution composed of 5% phenol (30 ml), 1% methylene blue (2 ml), and acetic acid (8 ml). The slides are processed through alcohol and xylol and are then mounted. A known area of the slide is scanned and the cells in the area counted. The total number in the soil can then be estimated. This technique has given high counts of Testacea (Heal, 1964; Volz, 1964) and, with staining, has enabled distinction between living and dead Testacea (Heal, 1964). Further experimentation is needed for recognizing and counting unshelled Protozoa.

An alternative method, which has produced high counts of Testacea only and is simpler and more efficient than the Jones and Mollison technique, has been described by Coûteaux (1967, 1975b). In this method a fresh soil sample (250 mg) is fixed in 1 ml of Bouin-Hollande solution[2] for a minimum of 24 hours, stained with 3 ml of 1% xylidine ponceau 2R (Aldrich Chemical Co., Milwaukee, Wis.; Sigma Chemical Co., St. Louis, Mo.) for 30 min, and diluted with distilled water to a volume of 250 ml. The soil material is disrupted by agitation to release tests intimately associated with the soil particles. A small subsample (5 ml) of the suspension is then filtered through a Millipore filter (diam 25 mm, pore size 0.45 μm) in a filtering system connected to vacuum pressure. The filter is air-dried, cleared in xylene, and mounted in Canada balsam. Testacea are then counted microscopically ($\times$ 250 magnification under phase contrast) by examining known areas of the filter or the whole filter. The staining involved in this technique allows differentiation between empty tests and active cells (Coûteaux, 1967) and encysted cells (Lousier, 1974a, b; 1975a, b; 1979[1]). It is undesirable to establish absolute instructions for the use of Coûteaux preparations for all litter and soil layers because of natural variations in testacean communities and populations and in litter and soil types. It is recommended, therefore, that the general method be used, but modifica-

[2] Bouin-Hollande solution: Combine 6.25 g of copper acetate [Cu(OAc)] with 250 ml of distilled water. Add 10 ml of picric acid, and filter. Then add 25 ml of 40% formalin and 2.5 ml of glacial acetic acid.

tions (based on preliminary experimentation) should be used to enable the maximum quantitative as well as qualitative data to be obtained (Lousier, 1979).[1]

The use of the Coûteaux preparations was investigated by Lousier (1979)[1] in testacean population studies in a cool temperature deciduous forest soil by evaluating the number of samples and sample replication required, and the type and length of sample maceration required, and by comparing this technique to the agar film technique used by Heal (1964). The sample and maceration tests were part of preliminary technical experimentation carried out before the beginning of extensive population studies. The results may be peculiar to this soil and testacean community, but comparison of the techniques shows that the number of species observed, the number of active and encysted Testacea and empty tests counted for each population, and the number of Testacea were substantially and significantly greater using the Coûteaux technique (Tables 52–4 to 52–6) despite the preparation and examination of only one-tenth the number of slides prepared and examined with the agar film technique.

The Coûteaux technique allowed 20 to 40 times more sample per unit area to be examined (i.e., 2 to 4 $mg \cdot cm^{-2}$ vs. 0.1 $mg \cdot cm^{-2}$ for the agar films), and 50 to 100 times more sample per slide (i.e., 5 to 10 mg but only 0.1 mg in the agar films). This reduced considerably the number of replicate slides necessary and saved time in sample processing and examination. Because the agar film technique requires particles that do not exceed 100 μm (Heal, 1964), prolonged maceration was needed. Even with the longer maceration time, there was still a large number of particles >100 μm, especially in the L and F layers. These larger particles accumulated around the edge of the hemacytometer platform used and prevented smaller particulate material from entering. This barrier may have prevented many Testacea, particularly the large species, from being drawn into the hemacytometer chamber. Perhaps in less organic layers and soils, where maceration or grinding can reduce most particles <100 m, the agar film technique could be used to estimate the total number of Testacea. For population or community studies in forest soils and in organic soils in general, it appears that the agar film technique may be inadequate.

Table 52–2. Range of estimates of numbers of naked protozoa in several soils and rhizospheres by dilution method.

Soil	Protozoa $\times 10^3 \cdot g^{-1}$ dry wt			Author
	Amoebae	Flagellates	Ciliates	
		Cultivated cropland		
Unplanted soil	10.58–50.05	0.57–13.95	0.03–0.28	Darbyshire & Greaves (1967)
Rhizosphere	18.05–180.87	1.60–65.22	0–1.75	Darbyshire & Greaves (1967)
		Subtropical forests		
Soil	14.80–823.15	22.04–3127.50	0.01–132.63	Bamforth (1976)
Rhizosphere	1.12–30750.00	4.07–823.15	0.28–132.63	Bamforth (1976)

Table 52-3. Range of estimates of numbers of Testacea in various soils.

Soil	$\times 10^3 \cdot g^{-1}$ wet wt	$\times 10^3 \cdot g^{-1}$ dry wt	$\times 10^6 \cdot m^{-2}$	Author
Dilution methods				
Coniferous litter and soil	1.6–24.0			Bamforth (1971)
Deciduous litter and soil	0.4–19.0			Bamforth (1971)
Coniferous-deciduous litter and soil	2.2–10.0			Bamforth (1971)
Coniferous-grassland litter and soil	0.4–8.0			Bamforth (1971)
Grassland litter and soil	1.0–14.0			Bamforth (1971)
Direct, agar films				
Woodland brown earth, mull			13–19	Volz (1964)
Woodland brown earth, moder			484–491	Volz (1964)
Woodland			5–25	Volz (1964)
Humus under oak and *Vaccinium*	17.7	46.0		Coûteaux (1967)
Beech and pinewoods on sand			5–25	Schönborn (1962)
Grassland, mineral		9–69	450–900	Heal (1965)
Woodland brown earth mull and moder		2–73	190–1,050	Heal (1964, 1965)
Direct, Millipore filters				
Burnt savannah				
Litter under vegetation clumps			1.22	Coûteaux (1976)
Rooting zone under clumps			1.25	Coûteaux (1976)
Surface of bare soil			0.92	Coûteaux (1976)
Rooting zone under bare soil			0.79	Coûteaux (1976)
Sphagnum in bogs and fens			9.2	Meisterfeld (1977)
Antarctic moss-turf peat, 0–3 cm			0.01–0.07	Smith (1973)
Antarctic moss-turf peat, 3–6 cm			0.009–0.075	Smith (1973)
Antarctic moss-turf peat, 6–9 cm			0.006–0.094	Smith (1973)
Humus under oak and *Luzula*		4.5–40		Coûteaux (1967)
Coniferous forest humus		11.5–46		Coûteaux (1975a, b)
Humus under oak and *Molinia*		1.5–27		Coûteaux (1975a, b)
Sub-Antarctic soils		1.4–15.9		Smith (1975)
Aspen woodland, Luvisol				
Litter layer		3.2–39.3	3.9–49.5	Lousier (1979)†
Fermentation layer		4.6–140.6	10.3–378.6	Lousier (1979)†
Humus layer		2.6–170.5	13.4–580.1	Lousier (1979)†
Black mineral layer		0.4–20.3	10.6–300.2	Lousier (1979)†
Direct, production chambers				
Beech forest moss			1.7	Schönborn (1977)
Beech forest soil			360.0	Schönborn (1975)
Coniferous forest soil			48.8	Schönborn (1975)

(continued on next page)

Table 52-3. Continued.

Soil	$\times 10^3 \cdot g^{-1}$ wet wt	$\times 10^3 \cdot g^{-1}$ dry wt	$\times 10^6 \cdot m^{-2}$	Author
		Direct, stained smears		
Spruce Podzol			6.2–6.7	Korganova & Geltser (1977)

† J. D. Lousier. 1979. Population ecology of Testacea (Protozoa, Rhizopoda) in an aspen woodland soil. Ph.D. Thesis. Univ. of Calgary, Calgary, Alberta, Canada.

The only difficulties encountered using the Millipore filtering technique result from a reduction in microscopic resolution by the filters. Because of this, accurate identification of species similar in appearance is sometimes difficult (Lousier, 1975a; 1976). Good microscopic resolution is required also to ascertain the location and extent of the cyst protoplasm and the appearance of the epiphragm in some of the encysted forms, particularly those specimens < 45 μm long.

The Coûteaux technique has proved to be a practical, direct observation technique that enables a realistic identification and evaluation of the testacean community and a better estimate of the abundance and activity of Testacea in litter and soil and has proved to be a technique that can be used for comparative population and community studies of Testacea in different soils and different soil layers. A similar technique has been developed for motile protozoa (Ehrau et al., 1969), but requires further evaluation.

Korganova and Geltser (1977) described another direct observation technique that gave them higher estimates of numbers of Testacea in comparison with the soil suspension technique of Volz (1951). Their method is a modification of stained smears used for bacteria and fungi. A fresh sample (5 g) is soaked in water (50 ml) for several hours; this well-washed suspension is then shaken for 10 min. After the suspension is shaken, 0.05 ml (containing 5 mg of sample) is withdrawn and mixed thoroughly with a drop of 0.5% agar on a microscope slide, the mixture then being spread over a known, suitable area. The smear is air-dried and stained with 1% erythromycin (Erythrocin, Abbott Laboratories, North Chicago, Ill.) solution in 5% carbolic acid for 1 hour. Excess stain is washed off in a series of water baths and the smear is air-dried again. The whole smear (or only portions of the smear) is examined under $\times$ 400 magnification.

Korganova and Geltser (1977) found that the smears provided a two-fold and a 10-fold increase in number of species observed and number of Testacea counted, respectively, over the direct observation technique of Volz (1951). They attributed the difference to the ability of the smear technique to reveal smaller organisms in greater numbers, but they provided no indication of which species these were and what type of soil was studied. Difficulties were encountered in distinguishing some species because of their orientation on the smear and the effects of the agar on microscopic resolution, but this technique appears to have less control over the thickness of the agar film than the Jones-Mollison technique and its accuracy has yet to be assessed statistically.

Table 52–4. Total number of Testacea counted from each layer of each soil core (i.e., each figure represents the total counts for four slides) by the Millipore filtering technique.

Species	Empty tests				Active forms				Encysted forms			
	L	F	H	Ah	L	F	H	Ah	L	F	H	Ah
Arcella catinus	4	2	2	--	1	--	--	--	--	--	--	--
Centropyxis aerophila	3	15	8	--	2	1	1	2	--	1	--	--
C. aerophila var. *sphagnicola*	4	17	8	--	2	1	2	2	--	1	--	--
C. minuta	--	1	1	--	--	--	--	--	--	--	--	--
C. plagiostoma	--	1	2	3	--	--	--	--	--	--	--	--
C. platystoma	--	--	1	--	--	--	--	--	--	--	--	--
C. cassis	--	--	--	4	--	--	--	--	--	--	--	--
Cyclopyxis eurystoma	3	5	2	--	--	--	--	--	--	--	--	--
C. eurystoma var. *gauthieriana*	1	1	--	--	--	--	--	--	--	--	--	--
C. kahli	--	--	1	--	--	--	1	--	--	--	--	--
Plagiopyxis callida	--	--	--	2	--	1	1	2	--	--	--	--
Hyalosphenia minuta	6	15	4	--	1	1	2	--	1	--	--	--
H. subflava	2	6	--	--	--	1	--	--	--	--	--	--
Heleopera petricola	3	10	--	--	1	2	--	--	1	--	--	--
Phryganella acropodia	13	41	57	20	2	10	2	4	2	2	--	2
Difflugiella oviformis	7	--	25	6	2	5	6	2	2	--	--	--
Trinema enchelys	10	143	58	13	2	30	6	2	3	8	3	1
T. lineare	13	100	34	14	1	26	14	3	3	9	3	2
Euglypha rotunda	7	34	33	11	1	7	3	1	1	2	--	--
E. laevis	9	54	31	9	1	10	3	--	1	1	--	--
E. scutigera	--	1	9	5	--	1	2	1	--	--	--	--
Totals	85	446	276	87	15	96	43	18	14	24	6	5
Number of species	14	16	16	10	11	13	12	8	8	7	2	3

Table 52–5. Total number of Testacea counted from each layer of each soil core (i.e., each figure represents the total counts from 40 slides) by the agar film technique.

	Empty tests				Active forms			
Species	L	F	H	Ah	L	F	H	Ah
Arcella catinus	1	--	--	--	--	--	--	--
Centropyxis aerophila	1	1	--	--	--	1	--	--
Hyalosphenia minuta	--	--	1	--	--	--	--	--
Heleopera petricola	--	--	--	--	--	1	--	--
Trinema enchelys	--	--	1	--	--	1	1	--
Euglypha laevis	--	1	--	--	--	--	--	--
Totals	2	2	2	0	0	3	1	0
Number of species	2	2	2	0	0	3	1	0

Table 52–6. Comparison of the estimates of testacean abundance (± standard error) by the Millipore filtering technique and the agar film technique.

		Numbers of Testacea (g^{-1} dry layer)	
Testacean life form	Soil layer	Millipore filtering technique	Agar film technique
Empty tests	L	4,592 ± 820	5,900 ± 4,859
	F	46,605 ± 6,918	5,235 ± 4,865
	H	29,967 ± 4,466	5,928 ± 5,427
	Ah	5,000 ± 959	0
Active forms	L	810 ± 211	0
	F	10,031 ± 962	7,837 ± 5,984
	H	4,668 ± 1,007	2,932 ± 2,928
	Ah	1,034 ± 414	0
Encysted forms	L	757 ± 267	0
	F	2,508 ± 558	0
	H	651 ± 182	0
	Ah	402 ± 83	0

52–3.3 Dilution Methods

All dilution counting techniques rely on the MPN method (section 39–3.3). They vary in the diluent, the degree of dilution, and the number of replicates for each dilution culture. Originally the dilution was cultured in a test tube and a sample pipetted out for the determination of growth or sterility, but a major advance was made by Singh (1946, 1975), who used agar plates and tested each dilution within a ring of bacterial inoculum on a plain agar plate.

Polypropylene or glass rings may be embedded in agar or in glass cups with agar in the bottom placed in petri dishes. Eight rings or cups, each containing 0.5 ml of dilution, are used for each of 15 levels in Singh's method; 10 rings or cups, each containing 1.0 ml of dilution, are used for each of three levels in Stout's (1962) modification.

In Singh's method, the initial dilution is 1:5, from which a series of twofold dilutions are made (1:10, 1:20, etc.). Stout's modification is 10-fold; 10^1 to 10^3 dilutions for nutrient-poor soils, 10^2 to 10^4 for moist soils and hard-leafed litters, and 10^3 to 10^5 or higher for humid soft-leafed

litters. Most flagellates and ciliates and many amoebae appear in the first week, but addition of *Enterobacter aerogenes* in the second week induces remaining encysted amoebae to appear. The number of negative rings are counted and compared with the appropriate table to estimate numbers (Singh, 1946; Stout, 1962; Fisher & Yates, 1943). Statistically, the twofold dilution series is more precise than the 10-fold dilution series but requires four times as many as cultures (Stevens, 1959). The method chosen is effectively determined by the resources available and the purpose of the investigation.

The use of small cups enables a larger quantity of dilution to be added and prevents desiccation. The use of a bacterial inoculum for food normally is not necessary, since bacteria will be present in the soil dilution, but it ensures a suitable and adequate food supply. By pretreating the soil with HCl (2%), unencysted protozoa will be killed, and the technique will give an estimate of encysted protozoa.

A number of modifications and improvements have been made to this technique and should be studied before it is applied to any particular soil (Darbyshire et al., 1974; Rowe et al., 1977). Its chief disadvantage, particularly with the bacterial ring technique, is that it estimates the bacteriophagous populations, which form only part of the biomass.

52–4 MICROSCOPIC EXAMINATION

52–4.1 Introduction

Because they are normally so small and are directly associated with soil particles or organic debris, protozoa have not been readily seen by direct observation. However, modern light microscopy with critical incident light illumination has made direct observation more feasible (Cassida, 1976; Wessenberg & Reed, 1971). Similarly, the preparation of gelatin-impregnated soil sections or thin sections of resin-impregnated soil has permitted direct observation of protozoa in situ (Haarlov & Weis-Fogh, 1953; Burges & Nicholas, 1961; Heal, 1964). Although hitherto restricted to bacteria and fungi, the fluorescent antibody technique is also applicable to soil protozoa (Hill & Gray, 1967; Schmidt, 1974).

Unfortunately, although various direct methods have been tried, e.g., soil suspension in water (Schönborn, 1962; Varga, 1959; Volz, 1951), soil suspension in agar (Bunt & Tchan, 1955), prepared soil sections (Alexander & Jackson, 1955; Haarlov & Weis-Fogh, 1953; Minderman, 1956), soil in agar films (Heal, 1964; Volz, 1964), soil on Millipore filters (Millipore Corp., Bedford, Mass.) (Coûteaux, 1967, 1975b), and stained soil smears (Korganova & Geltser, 1977), none has been rigorously tested, widely used, or shown to be applicable to a wide range of protozoa. Most of these methods have proven useful for Testacea with their resistant tests, but preparation of the soil for examination usually makes other protozoa unrecognizable or unidentifiable (Heal, 1971).

An entirely new chapter of microscopy has been opened with electron

microscopy. The development of the scanning electron microscope (SEM) and its three-dimensional image not only has immensely extended the resolution of the microscope but also has restored a measure of reality to microscopic study. The continuing development of methods of preparation and examination provides the best prospect of reevaluation of the place of protozoa in soil studies.

52-4.2 Light Microscope

Protozoa must be examined in the living condition, although fixation and staining may be necessary to confirm identification. Easily recognized forms can be identified in cultures, but often they must be transferred to single drops on slides for further study and for staining.

52-4.2.1 LIVE CELLS

A convenient summary of methods is given by MacKinnon and Hawes (1961), and microscopical techniques and formulas are given by Gray (1954). The development of phase and interference contrast as well as the wide range of lenses now available has greatly facilitated the examination and identification of the protozoa (Plachter, 1979). Cooling, or such preparations as methyl cellulose, 1% $NiSO_4$, or protamine (Marsot & Couillard, 1973), may be used to slow the motile cells of the flagellates and ciliates on wet slides or in hanging drops, and vital stains can be used to determine flagella, cilia, nuclei, and other organelles. There is, however, no substitute for patient observation, and techniques have been developed for prolonged observations (Schönborn, 1977; Tuffrau, 1964, 1967b; Page, 1976).

52-4.2.2 FIXED CELLS

Both Bouin's and Schaudin's fixatives are convenient all-purpose fixatives, but the best results are obtained with osmic acid. For precise identification, it is generally necessary to have the exact dimensions and the details of the principal organelles. For amoebae, it is necessary to determine the mitotic pattern (Singh, 1975; Page, 1976). In the case of ciliates, the "silver line" system, revealed by either the Klein dry silver technique or the Chatton-Lwoff wet silver technique (Corliss, 1953, 1961), provides definitive taxonomic criteria for most species. However, it is not always readily revealed, and other techniques, such as Protargol (Winthrop Laboratories, New York) (Dragesco, 1962; Tuffrau, 1964, 1967b), may have to be employed.

52-4.2.3 PERMANENT PREPARATIONS

Fixed and stained cells (Korganova & Geltser, 1977) mounted in permanent preparations are generally very small and difficult to find without being ringed or located in some other way. Permanent preparations are important since they are required as type material for descriptions of new

species and must be deposited in a type collection, such as the American Type Culture collection or the British Museum of Natural History, when a new species description is to be published. They are also useful as a working collection to help identification.

52–4.3 Electron Microscope

52–4.3.1 TRANSMISSION ELECTRON MICROSCOPY (TEM)

For very small cells, such as flagellates, it is possible to mount the whole cell for examination, but normally the cells must be sectioned. Although this is necessary for detailed cytological studies, it is not normally required for identification.

52–4.3.2 SCANNING ELECTRON MICROSCOPY (SEM)

Although the SEM system reveals only the surface architecture of the organism, this is often sufficient to determine critical morphological characters. In Testacea, these have included structure of the test, pseudostome structure and position, aperture platelets, spines, pseudopodial form, the arrangement and attachment of the cell to the test, and the sequential formation of the new test during cell division (Hedley & Ogden, 1973, 1974a, b; Hedley et al., 1974; Netzel, 1971, 1972a–c, 1975a, b, 1976, 1977; Moraczewski, 1970, 1971; Kies, 1974). In ciliates, they have included surface cilia pattern and organelles (Small et al., 1971b) and binary fission (Small et al., 1971a). Preparation is easier than that for the TEM (Harris et al., 1972; Small & Marszalek, 1969), because the complexities and precision required for sectioning are avoided, and the three-dimensional image is invaluable in determining morphological relationships (Harris et al., 1976). The basic preparation (Small & Marszalek, 1969; Lousier, 1972[3]) involves fixing washed specimens in a fixative, such as Parducy's (OsO_4 and $HgCl_2$), for 5 to 10 min, placing rewashed, fixed specimens onto SEM stubs, and freezing the specimens in liquid N. Under vacuum and cold temperature, the specimens are dried for 5 hours. At room temperature and high vacuum, the specimens are coated with Au or a Au–Pd alloy. The specimens are then ready for observation under the SEM.

The use of an electron probe in conjunction with the SEM enables the identification of a range of elements, particularly revealing in the case of testacean shells (Jeanson & Coûteaux, 1977; Stout & Walker, 1976).

52–5 CONCLUSIONS

Free-living protozoa constitute a small but significant part of the microbial population of most soils. Current opinion sees their prime importance as micropredators serving to accelerate nutrient turnover. Thus, apart from the relative numbers of the different groups in different soils,

[3] J. D. Lousier. 1972. Taxonomy and ecology of Testacea (Rhizopoda) in Rocky Mountain soils (Kananaskis Valley, Alberta, Canada). M.Sc. Thesis. Univ. of Calgary, Calgary.

attention is directed to their biomass and to their metabolic activity (Heal, 1971). The original enumeration data, gathered very laboriously by dilution counts, failed to consider either of these aspects. What are currently required are simpler and more direct techniques that will provide information on the rapid population fluxes that occur in soil either in response to seasonal or cultural changes or by the redistribution of energy between all populations. The Coûteaux technique and the application of SEM to soil provide the two most positive advances of recent years, but they have been of most value in the study of the Testacea, only one of the groups of free-living protozoa. The weakness of the dilution (MPN) technique lies not in its statistics but in its biology. How reliable is the culture technique in determining the number of viable cells in the soil? What is lost in the dilution process? Are flagellates and amoebae the most numerous MPN protozoa simply because they are less susceptible to dilution shock and more easily fed than ciliates? Calibration of MPN against direct counting—possibly by fluorescent antibody technique—is a current challenge to soil protozoologists. Finally, how convincing is the turnover data based simply on population estimates? Integration of population estimates with other measures of cell activity could well help to resolve this dilemma (Kaszubiak et al., 1977).

The era of faunal lists and population estimates is past; attention must be directed toward population dynamics rather than toward standing crops. This will mean greater specialization protozoologically, since it presumes an intimate knowledge of the protozoa involved, but it should lead to a better integration in soil microbiology. It is in this region that the tools are now available to determine far more precisely than hitherto the life of the protozoa in the soil.

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